Mammalian Na⁺/Ca²⁺ exchangers are members of three branches of a much larger family of transport proteins [the CaCA (Ca²⁺/cation antipporter) superfamily] whose main role is to provide control of Ca²⁺ flux across the plasma membranes or intracellular compartments. Since cytosolic levels of Ca²⁺ are much lower than those found extracellularly or in sequestered stores, the major function of Na⁺/Ca²⁺ exchangers is to extrude Ca²⁺ from the cytoplasm. The exchangers are, however, fully reversible and thus, under special conditions of subcellular localization and compartmentalized ion gradients, Na⁺/Ca²⁺ exchangers may allow Ca²⁺ entry and may play more specialized roles in Ca²⁺ movement between compartments. The NCX (Na⁺/Ca²⁺ exchanger) [SLC (solute carrier) 8] branch of Na⁺/Ca²⁺ exchangers comprises three members: NCX1 has been most extensively studied, and is broadly expressed with particular abundance in heart, brain and kidney, NCX2 is expressed in brain, and NCX3 is expressed in brain and skeletal muscle. The NCX proteins subserve a variety of roles, depending upon the site of expression. These include cardiac excitation–contraction coupling, neuronal signalling and Ca²⁺ reabsorption in the kidney. The NCKX (Na⁺2/Ca²⁺–K⁺ exchanger) (SLC24) branch of Na⁺/Ca²⁺ exchangers transport K⁺ and Ca²⁺ in exchange for Na⁺, and comprises five members: NCKX1 is expressed in retinal rod photoreceptors, NCKX2 is expressed in cone photoreceptors and in neurons throughout the brain, NCKX3 and NCKX4 are abundant in brain, but have a broader tissue distribution, and NCKX5 is expressed in skin, retinal epithelium and brain. The NCKX proteins probably play a particularly prominent role in regulating Ca²⁺ flux in environments which experience wide and frequent fluctuations in Na⁺ concentration. Until recently, the range of functions that NCKX proteins play was generally underappreciated. This situation is now changing rapidly as evidence emerges for roles including photoreceptor adaptation, synaptic plasticity and skin pigmentation. The CCX (Ca²⁺/cation exchanger) branch has only one mammalian member, NCKX6 or NCLX (Na⁺/Ca²⁺–Li⁺ exchanger), whose physiological function remains unclear, despite a broad pattern of expression.

Key words: calcium transport, excitation–contraction coupling, melanosome biogenesis, renal calcium reabsorption, signal transduction, sodium/calcium exchanger, synaptic plasticity.

INTRODUCTION

Ionized calcium (Ca²⁺) is utilized by cells as a ubiquitous signalling molecule, and changes in its concentration control a variety of diverse physiological events from the beginning of life to its end, as well as at many points in between. It is now widely appreciated that specificity in Ca²⁺ signalling is achieved largely through complex spatial and temporal dynamics brought about by the integrated action of a large set of channels and transporters that move Ca²⁺ across membranes between different compartments. Alteration in the function or regulation of any of these carefully choreographed players has serious deleterious consequences, often associated with the pathogenesis of disease [1,2].

Na⁺/Ca²⁺ EXCHANGERS: FROM DISCOVERY TO THE MOLECULAR ERA

Among the different pathways that mediate Ca²⁺ movement, Na⁺/Ca²⁺ exchange has emerged as the predominant mechanism for Ca²⁺ efflux across the plasma membrane, particularly when overall Ca²⁺ levels are high [3–5]. Moreover, several recent studies have highlighted the connection between important physiological events and specific Na⁺/Ca²⁺ exchanger molecules [6–8]. Na⁺/Ca²⁺ exchange was first described in squid axon and mammalian heart some four decades ago. Extensive studies demonstrated that Na⁺/Ca²⁺ exchange played a crucial role in Ca²⁺ extrusion, and operated with a stoichiometry of three Na⁺ ions to one Ca²⁺ ion, indicating that the transport process was electrogenic [9]. The development of partially purified exchanger preparations, antibody reagents and expression cloning techniques led to the molecular cloning of the canine cardiac Na⁺/Ca²⁺ exchanger, subsequently denoted NCX1 [10]. The NCX1 cDNA encoded a protein of 970 amino acids predicted to be approx. 110 kDa in size. The mature protein is subject to both signal peptide cleavage and glycosylation, and, when analysed on gels, runs with an apparent size of 120 kDa. Additional bands are observed at 160 and 70 kDa, which arise by heat-induced aggregation and proteolytic cleavage respectively. Further minor variations in apparent size (5–10 kDa) are generated by alternative splicing.

Parallel studies in retinal rod photoreceptors had established the presence there of a mechanistically similar Na⁺/Ca²⁺-exchange process that is critical for visual adaptation and represents the predominant means of Ca²⁺ extrusion from rod outer segments [11]. The rod exchanger differed from the one described in heart and axon principally due to its absolute transport requirement for K⁺ ions. These studies indicated that the rod exchanger catalysed the transport of 4 Na⁺ in exchange for 1 Ca²⁺ and 1 K⁺ [12,13]. Purification of the bovine rod Na⁺/Ca²⁺–K⁺ exchanger

Abbreviations used: CaCA, Ca²⁺/cation antipporter; CAX, Ca²⁺/anion exchanger; CBD, Ca²⁺-binding domain; CCX, Ca²⁺/cation exchanger; DREAM, downstream regulatory element antagonist modulator; HEK-293, human embryonic kidney; LTP, long-term potentiation; NCKX, Na⁺/Ca²⁺–K⁺ exchanger; NCLX, Na⁺/Ca²⁺–Li⁺ exchanger; NCX, Na⁺/Ca²⁺ exchanger; PIP₂, phosphatidylinositol bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLM, phospholemman; SLC, solute carrier; SR, sarcoplasmic reticulum; XIP, exchanger inhibitory peptide.

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subsequently led to its molecular cloning [14,15]. This cDNA, denoted NCKX1, encoded a protein of 1216 amino acids predicted to be approx. 132 kDa in size. Extensive glycosylation of the protein appears to account for the much larger apparent size of the native protein observed on gels (> 220 kDa). Despite their obvious mechanistic and architectural similarities, NCX1 and NCKX1 share only very limited sequence identity, within a duplicated motif referred to as the α-repeat (see below).

A SUPERFAMILY OF Na⁺/Ca²⁺ EXCHANGERS

These initial molecular cloning events led to a rapid explosion of sequence information on related molecules that were isolated by low-stringency library screening or database mining. Soon it was established that NCX1 was part of larger gene family, SLC (solute carrier) 8, whereas NCKX1 was the founding member of another family, SLC24. More recent analysis has established that SLC8 and SLC24 are both part of a larger superfamily of related Ca²⁺/cation antipporter genes, the CaCA superfamily [16–18] (see Figure 1). This superfamily has five major branches, tentatively named after characterized representative members: (i) YRBG, comprising largely bacterial members; (ii) CAX (Ca²⁺/anion exchanger), comprising mostly plant and yeast members; (iii) NCX (SLC8) and (iv) NCKX (SLC24), both comprising almost exclusively vertebrate members; and (v) CCX (Ca²⁺/cation exchanger), which contains the partially characterized molecule previously called NCKX6 or NCLX (Na⁺/Ca²⁺/Li⁻ exchanger). The YRBG and CAX branches of the family probably catalyse Na⁺/Ca²⁺ exchange; NCX and NCKX, of course, catalyse Na⁺/Ca²⁺ exchange, and the CCX family has been demonstrated to catalyse both Na⁺/Ca²⁺ exchange and Li⁻/Ca²⁺ exchange, but, by virtue of its phylogeny in a separate branch, may have an as yet undetermined endogenous specificity. Mammals express three members of the NCX (SLC8) family, NCX1, NCX2 and NCX3 [10,19,20] (a new fourth member of the NCX family has recently been identified in teleost fish [21], but is not present in mammalian genomes); five members of the NCKX (SLC24) family: NCKX1, NCKX2, NCKX3, NCKX4 and NCKX5 [15,22–25]; whereas NCKX6/NCLX is the lone member of the CCX branch found in mammals [26,27].

The CaCA superfamily of transporters is characterized by a conserved overall membrane topology comprising two clusters of five hydrophobic putative transmembrane helices, joined by a cytoplasmic loop of varying length. Certain members of the family appear to possess, in addition, a cleaved putative signal peptide at the N-terminus and extra (or sometimes fewer) putative transmembrane spans in each hydrophobic cluster [17] (Figure 1). In addition to this characteristic membrane topology, the CaCA superfamily is also defined by the presence of a signature conserved amino acid sequence motif present within each hydrophobic cluster, the so-called α-repeat regions [16]. Residues within these regions have been demonstrated through mutagenesis experiments to be critical for the transport function of both NCX1 [28–31] and NCKX2 [32,33], and are thought to form the ion-binding pocket of these exchangers through the physical association of two α-repeats, one from each hydrophobic cluster, oriented in opposite directions [29,34–39]. The two α-repeat regions together with the repeated pattern of hydrophobicity, indicate that these molecules each comprise a duplicated structure which presumably has its origins in a dimeric dual-topology membrane protein [40,41], although none has been identified thus far that is related specifically to the CaCA superfamily. The remainder of this review will focus on members of the CaCA superfamily that are expressed in mammals.

THE SLC8 Na⁺/Ca²⁺ EXCHANGER FAMILY

NCX1 is the most broadly expressed member of the SLC8 family. Originally characterized and cloned from heart, it was subsequently realized that NCX1 is also highly expressed in brain and kidney, and at lower levels in almost all other tissues [42–44]. Transcripts of the NCX1 gene are alternatively spliced at two sites in a tissue-specific manner [43,45]. The first site lies in the 5′-untranslated region and does not alter the structure of the encoded protein. Instead, the presence of different non-coding initiating exons is a consequence of three independent promoters selectively driving and regulating expression of NCX1 in different tissues: one specific to heart, one to kidney, and one for other tissues [46,47]. Presumably this situation provides the capacity to independently regulate gene expression in response to tissue-specific physiological demands.

Figure 1  CaCA phylogeny and Na⁺/Ca²⁺ exchangers

(A) Phylogenetic tree of 147 unique members of the CaCA superfamily is shown. Five major branches in the tree are labelled, and representative members are shown (GenBank® accession numbers are given in parentheses): EcoYRBG, the YRBG protein from Escherichia coli (NP_417663); CerVCX, the vacuolar Ca²⁺/H⁺ exchanger from Saccharomyces cerevisiae, (NP_010155); AthCAX1 and AthCAX2, Ca²⁺/H⁺ exchangers from Arabidopsis thaliana (NP_181352 and NP_566452); NCX1, NCX2 and NCX3, Na⁺/Ca²⁺ exchangers from Homo sapiens (NP_066920, NP_055878 and NP_150287); NCX1, NCX2, NCX3, NCX4 and NCX5, Na⁺/Ca²⁺/K⁺ exchangers from Homo sapiens (NP_084718, NP_050777, NP_054740, NP_170532 and NP_96532); NCLX/NCKX6 from Homo sapiens (NP_070235).

(B) Putative general topology model for proteins of the CaCA superfamily. Hydrophobic transmembrane helical segments are illustrated as cylinders and are linked to show the topological connectivity. The conserved α-repeat regions are shown boxed and oppositely oriented with respect to the membrane. The N-terminal transmembrane segment (light grey) is absent from some, and is thought to be removed by a co- or post-synthetic proteolytic cleavage event in other, members. The C-terminal two-transmembrane spans (light grey) are variably present in different family members. Additionally, and not shown, the CXX branch is predicted to have an additional hairpin pair of transmembrane helices between the central cytosolic loop and the α-2 motif. Modified from [17] with permission from Oxford University Press.
The membrane. The predicted size of the NCX2 and NCX3 protein C-terminus is thought to be on the intracellular side of the two which is followed by a hydrophobic region containing the first of protein is extracellular and part of a glycosylated region, translationally, and thus the N-terminus of the mature terminus acts as a ‘signal peptide’ and is cleaved co- or post- and supported by experimental evidence (see Figure 2). The N-terminus has been inferred indirectly from biochemical or mutagenic analyses. Aside from recent studies on part of the cytoplasmic loop (see below), information regarding the structure of NCX1 has largely been performed on NCX1, the conclusions are also likely to apply, at least in general terms, to NCX2 and NCX3. The three mammalian SLC8 family members display a high degree of sequence identity throughout the length of the protein. Thus, whereas almost all of the structural and functional work has been performed on NCX1, the conclusions are also likely to apply, at least in general terms, to NCX2 and NCX3. The second site of alternative splicing lies within the coding region of the transcript, where two mutually exclusive and four cassette exons encode a potentially large number of protein isoforms with variations in a cytosolic segment of the protein. The mutually exclusive exons appear to be incorporated such that transcripts in excitable tissues and cells (primarily muscle and nerve) include one exon (exon A), whereas transcripts in non-excitable cells and tissues include the other (exon B). No obvious tissue-specific pattern has been observed for the cassette exons. In contrast with NCX1, the other members of the SLC8 family have a much more restricted pattern of expression. NCX2 is abundant in neurons in all parts of the brain, but not present at significant levels elsewhere. NCX3 is expressed selectively in skeletal muscle and at lower levels in some brain regions. NCX2, but probably not NCX2, is alternatively spliced to generate variability in a similar cytoplasmic region as NCX1.

The precise topological folding arrangement of the polypeptide within the two hydrophobic membrane-embedded regions has been studied extensively. The current model proposes five transmembrane helices in the first hydrophobic cluster and four in the second for NCX1. The conserved α-repeats of NCX1 contain amino acids that are essential for function, including two critical acidic residues, one within each α-repeat [28–30]. Accessibility experiments indicate that the two α-repeats are oriented in opposite directions with respect to the membrane, with their central regions probably forming membrane re-entrant loops that have limited accessibility to both sides of the membrane. Cross-linking experiments have localized the helices predicted to flank the α-repeats in proximity with one another. Thus it seems most probable that the two α-repeats form part of a vestibule or pathway required for the binding and transport of ions through the NCX1 protein.

Not all the cross-linking data are compatible with the simple all-helical model for the two hydrophobic clusters, however. Instead, these data suggest that the α-repeats may form a more convoluted, intramembrane, structure which would be consistent with the presence of a number of helix-breaking residues present in these motifs. There is thus still much to be learned about the nature of the ion-binding sites in Na+/Ca2+ exchangers, including how one site can accommodate either one Ca2+ or three Na+ ions in a mutually exclusive manner, and why reorientation of the ion-binding site accessibility from one side of the membrane to the other requires its occupancy. It is possible that cation binding at the two key acidic amino acids is an essential step required to allow reorientation and transport. This model would be similar to the experimentally supported model for NCX1, which has nine proposed transmembrane-spanning segments (TMSs), a cleaved ‘signal peptide’ (TMS0, with the putative signal peptide cleavage site shown as SPase?) and re-entrant loops, one of which may be partially helical, as part of the conserved α-repeat motifs (dark blue, labelled α1 and α2). Critical amino acids in the α-repeat regions are highlighted in red (Glu111, Asp119, Asp404 and Asp492). The single N-linked glycosylation site is shown in green (N-CHO). Highlighted regions of the cytosolic loop include the XIP, the two CBDs (CBD1 and CBD2) and the region subject to alternative splicing. Proposed sites involved in regulatory Ca2+ binding are also shown.

**Figure 2** Topology model for NCX1

The experimentally supported model for NCX1 has nine proposed transmembrane-spanning segments (TMSs), a cleaved ‘signal peptide’ (TMS0, with the putative signal peptide cleavage site shown as SPase?) and re-entrant loops, one of which may be partially helical, as part of the conserved α-repeat motifs (dark blue, labelled α1 and α2). Critical amino acids in the α-repeat regions are highlighted in red (Glu111, Asp119, Asp404 and Asp492). The single N-linked glycosylation site is shown in green (N-CHO). Highlighted regions of the cytosolic loop include the XIP, the two CBDs (CBD1 and CBD2) and the region subject to alternative splicing. Proposed sites involved in regulatory Ca2+ binding are also shown.
in principle to that proposed for the \textit{Escherichia coli} GlpT transporter [57].

THE NCX1 CYTOPLASMIC DOMAIN

The cytoplasmic region is responsible for complex and physiologically important modes of NCX1 regulation, as well as being the target for several recently identified inhibitory compounds. Gaining structural insight into this critical region of NCX1 will thus provide a new opportunity to develop a mechanistic understanding of regulation and inhibition. Linearly, the central cytoplasmic loop of NCX1 can be broken down into different motifs (Figure 2). Beginning at the N-terminal end, adjacent to the first cluster of membrane-spanning helices, is an amphipathic sequence, called the ‘XIP’ (exchanger inhibitory peptide) region because exogenous addition of a peptide with this sequence inhibits exchanger function [10,58]. This region has been implicated in regulation of NCX1 function by both Na$^+$ and by acidic phospholipids (see below). The XIP region is followed by a sequence modelled to be similar to a catenin-like domain, then by two so-called $\beta$-repeats, originally identified as similar to $\beta$4 integrin [16], and more recently the subject of two structural studies [59,60]. It is now clear that each $\beta$-repeat together with flanking sequence corresponds to a CBD (Ca$^{2+}$-binding domain) with an immunoglobulin-like fold comprising a seven-stranded $\beta$-sandwich structure (see Figure 3).

The first of these domains (CBD1), binds four Ca$^{2+}$ ions with high affinity at one end of the $\beta$-sandwich [60], an event associated with a conformational change that is thought to be responsible for the known regulatory effect of Ca$^{2+}$ binding on NCX1 activity (see below). The second domain (CBD2) forms a structure homologous with CBD1 that binds Ca$^{2+}$ with lower affinity and without a corresponding large conformational change [59].

CBD2 also contains the region of variability owing to alternative splicing. The two CBDs are thought to pack together in an anti-parallel manner with the Ca$^{2+}$-binding end of CBD1 pointing into the cytoplasm away from the membrane [59]. The mutually exclusive alternatively spliced exons form a central and essential structural role in CBD2. However, the variable cassette exon portion of the alternatively spliced region resides in a loop of CBD2 that faces away from the membrane (see Figure 3). The C-terminal end of the cytoplasmic domain is composed of a hydrophobic and proline-rich sequence that was originally modelled as a transmembrane span. Subsequent experimental evidence indicates that this sequence actually lies on the cytoplasmic side of the membrane [34]. Despite its unusual sequence and somewhat conserved nature among other NC(K)X family members, no function for this region has yet emerged. The cytoplasmic loops of NCX2 and NCX3 display a relatively high degree of amino acid identity with that of NCX1, which indicates that the overall domain structure is likely to be conserved. Consistent with this view, both NCX2 and NCX3 display regulatory behaviour similar to that of NCX1 [61].

NCX1 TRANSPORT FUNCTION

Operation of the Na$^+$/Ca$^{2+}$ exchanger is fully reversible, and so the direction of ion movement depends entirely upon the electrochemical ion gradients and the number of ions that bind and are transported. For NCX1, the stoichiometry of ion transport has been investigated extensively for many years using a variety of techniques and a value of three Na$^+$ ions in exchange for one Ca$^{2+}$ ion has generally been accepted [9]. The resulting unequal movement of charge means that NCX1 transport is electrogenic [62], and thus can be measured using electrophysiology [63].

![Figure 3 Structural model for the NCX1 CBSs](http://www.biochem.jo/bcj/406/0365/bcj4060365add1.htm)

Several studies using ionic current to measure activity suggested a Na$^+$/Ca$^{2+}$ stoichiometry of 4:1 [64,65]. Although a rare (1 Ca$^{2+}$-1 Na$^+$) transport mode has been proposed to explain these apparent discrepancies in stoichiometry [66], it is not clear that the stoichiometry issue has been adequately resolved. Na$^+$/Ca$^{2+}$ exchange is thought to be mediated by a sequential series of binding, conformational changes and unbinding steps that involve one ion-binding site that can accommodate either three Na$^+$ ions or one Ca$^{2+}$ ion [except as proposed above for the rare (1 Ca$^{2+}$-1 Na$^+$) transport mode] [9]. Full occupancy of the site allows transport of the bound ion(s) across the membrane. This sequential mechanism, and the requirement for occupancy of the ion-binding site with either Na$^+$ or Ca$^{2+}$, accounts for the observation that the exchanger can catalyse Na$^+$/Ca$^{2+}$ exchange, as well as Na$^+$/Na$^+$ exchange and Ca$^{2+}$/Ca$^{2+}$ exchange [67] (see Figure 4).

Under normal resting cellular ionic conditions and membrane potential, NCX1 operating with a 3:1 stoichiometry will act to extrude Ca$^{2+}$ from the cytoplasm [9]. In the heart, during systole when the membrane becomes depolarized and Na$^+$ levels rise adjacent to the plasma membrane by flow through voltage-operated Na$^+$ channels, NCX1 reverses operation and allows a small amount of Ca$^{2+}$ entry [68]. This Ca$^{2+}$, while not directly triggering SR (sarcoplasmic reticulum) release and inducing contraction, may play an important regulatory role in the
excitation–contraction coupling process by influencing the gating of neighbouring voltage-operated Ca\(^{2+}\) channels and by altering the SR Ca\(^{2+}\) load. The functional interconnectivity of these different ion channels and transporters in the cardiac sarcolemma and SR is facilitated by the apposition of the two membrane systems, which creates a restricted diffusional, or ‘fuzzy’, space where ion concentrations can reach much higher levels than detected globally [69]. The importance of this physical arrangement in the context of excitation–contraction coupling is evident from the large physical protein complexes that normally exist here [70,71], and the serious consequences to heart function when they cannot form because of the absence of the scaffolding protein ankyrin [72]. The stoichiometry of ion transport has not been established experimentally for NCX2 or NCX3. On the basis of the high level of sequence identity within the putative transport regions, however, it is anticipated that these two proteins will behave identically with NCX1.

**REGULATION OF NCX1 FUNCTION**

Although the direction of Ca\(^{2+}\) flux through NCX1 depends upon the thermodynamic factors discussed above, the rate of transport depends upon kinetic factors, influenced by the amount of exchange protein present, the occupancy of the ion-binding sites and regulation of activity. Expression of the NCX1 gene in heart displays developmental regulation, being higher in neonatal animals and then declining gradually with age [73–76]. Such an expression pattern is consistent with the timing of SR and SR–T-tubule junction development, and the switch of cardiomycocyte Ca\(^{2+}\) handling from the plasma membrane to the SR [77]. NCX1 expression is also increased in cultured cardiomycocytes following α-adrenergic stimulation [46]. Otherwise, most studies examining transcriptional regulation of the NCX1 gene have focused on changes associated with pathological scenarios such as cardiac overload or end-stage heart disease, where expression of the molecule is generally up-regulated [78–81].

Regulation of the NCX2 and NCX3 genes has been studied in cell culture systems. When cerebellar granule cells are grown in the presence of high potassium concentrations, conditions which promote their differentiation, the expression of NCX2 is down-regulated via a Ca\(^{2+}\)/calciuneurin-dependent mechanism, while expression of NCX3 is up-regulated [82]. Further investigation of the regulation of NCX3 has revealed an important role for the Ca\(^{2+}\)-binding transcriptional regulator, DREAM (downstream regulatory element antagonist modulator). In this model, elevated [Ca\(^{2+}\)] binds DREAM, which relieves its action as a repressor, resulting in gene activation [83]. These observations suggest intriguing Ca\(^{2+}\)-dependent mechanisms for regulation of Ca\(^{2+}\) transporters and may explain expression of specific gene products in environments that experience large fluctuations in [Ca\(^{2+}\)].

The intracellular ion transport site of NCX1 has an apparent affinity for Ca\(^{2+}\) in the low micromolar range, and so the exchanger is not maximally activated until [Ca\(^{2+}\)] rises well above resting levels. This site also has an apparent affinity for Na\(^{+}\) of approx. 10–20 mM which is close to or a little higher than normal resting [Na\(^{+}\)]. The externally facing transport site has markedly different apparent affinities for Ca\(^{2+}\) and Na\(^{+}\) ions of approx. 1 and 60–80 mM respectively [63,84,85]. Thus the overall kinetic rate of the exchanger under normal resting cellular conditions is limited principally by occupancy of the cytoplasmic Ca\(^{2+}\)-binding sites. In addition to regulation by substrate levels, a number of other factors have been found to regulate NCX1 activity allosterically. These include Ca\(^{2+}\), Na\(^{+}\), PIP\(_{2}\) (phosphatidylinositol bisphosphate) and other acidic phospholipids, H\(^{+}\) and a variety of interacting ‘partner’ proteins [86].

Regulation of NCX1 activity by Ca\(^{2+}\) was first observed using dialysed squid axon, and then in patch-clamped myocytes and giant excised patches [63,87,88]. Measurement of the exchanger in the reverse, Ca\(^{2+}\)-entry, mode revealed the essential requirement of Ca\(^{2+}\) at the cytoplasmic surface for activity. Although as measured experimentally, Ca\(^{2+}\) activation of NCX1 is relatively slow [89,90], at least in principle this form of regulation might take place in a cardiac cell on a beat-to-beat basis [91]. Treatment of the excised patches with chymotrypsin removes Ca\(^{2+}\) regulation, leading to constitutive activation of the exchanger [89]. Acidic clusters within the central cytosolic loop of the exchanger were shown later to be critical for Ca\(^{2+}\) regulation [92,93] and, as presented above, most recently the CBDs have been characterized structurally [59,60]. Although evidence for conformational changes of these domains in response to Ca\(^{2+}\) binding has been

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**Figure 4 Proposed transport cycle for Na\(^{+}\)/Ca\(^{2+}\) exchangers**

(A) The NCX1 cycle. The empty exchanger is shown to bind either one Ca\(^{2+}\) ion or three Na\(^{+}\) ions, in a mutually exclusive manner. Either binding event allows a conformational change that reorients the accessibility of the ion-binding site across the membrane barrier. Forward-mode Ca\(^{2+}\) extrusion in exchange for Na\(^{+}\) entry occurs by sequential steps in a clockwise direction. Conversely, reverse-mode Ca\(^{2+}\) influx in exchange for Na\(^{+}\) exit proceeds around the cycle in an anti-clockwise direction. (B) The NCX2 cycle. As in (A), except the empty transporter binds either one Ca\(^{2+}\) ion plus one K\(^{+}\) ion or four Na\(^{+}\) ions. The binding of Ca\(^{2+}\) and K\(^{+}\) is shown ordered based on unpublished work (F. Visser and J. Lytton) and because kinetic considerations under normal physiological ion concentrations suggest that the cycle is likely to be limited by Ca\(^{2+}\) binding to an exchanger with a cytoplasmically oriented site primed with bound K\(^{+}\). An animated version of this Figure is available at http://www.BiochemJ.org/bj/406/0365/bj4060365add2.htm.
measured both biophysically and biochemically, a mechanistic model to explain regulation of exchanger activity is still lacking. Curiously, the Drosophila melanogaster orthologue of NCX1 (CALX1) is inhibited, rather than activated, by Ca$^{2+}$ binding to a similar region [94]. Chimaeric constructs revealed that the stimulatory property of Ca$^{2+}$ binding could be re-introduced partially into CALX1 together with a region encompassing CBD1 from mammalian NCX1 [95].

While cytosolic Ca$^{2+}$ activates the exchanger, high levels of cytosolic [Na$^+$] have been shown to inhibit activity [89,96]. This so-called Na$^{+}$-dependent inactivation is observed as a significant decline to a steady-state level following the rapidly developing current that declines with time to a steady-state level is measured when Na$^+$ is applied to activate NCX1 transport in excised patches. Na$^+$-dependent inactivation was originally modelled to take place following Na$^+$ binding to the cytoplasmically oriented transport site, via a transition to an inactive conformation of the exchanger [96]. An alternative model has also been proposed, in which Na$^+$ interacts with a regulatory site distinct from the transport site [86]. Mutations in the amphipathic XIP region at the N-terminus of the cytosolic domain have been shown to have a major influence on Na$^{+}$-dependent inactivation, some mutations preventing inactivation, and some enhancing it [97].

ATP was recognized to be an important activator of NCX1 when measured using the giant excised patch preparation [89,98]. The ATP effect is predominantly as a consequence of preventing Na$^{+}$-dependent inactivation, and appears to involve the production of the phosphorylated phospholipid, PIP$_2$ [99]. Further investigation of the mechanism revealed that the XIP region was involved in PIP$_2$ activation, as might be expected based on the interaction of this domain with Na$^{+}$-dependent inactivation [100]. Key mutations in the XIP region have reciprocal effects on Na$^{+}$-dependent inactivation and PIP$_2$ activation. NCX1 has also been demonstrated to be regulated by a variety of other lipids [101–104]. A recent intriguing report suggested that NCX1 in heart may be regulated in a manner co-ordinated with fat metabolism via activation by long-chain acyl-CoA esters [105]. This activation was shown to involve the XIP region, in a manner analogous to activation by PIP$_2$, although the mechanism appeared to be different.

Na$^{+}$-dependent inactivation of NCX1 can also be prevented by raising pH [106], or by increasing Ca$^{2+}$ to levels higher than those required for Ca$^{2+}$ activation [98]. The alleviation of inactivation by Ca$^{2+}$ is only observed in those NCX1 isoforms containing the mutually exclusive alternatively spliced exon A (i.e. NCX1.1, NCX1.4 and NCX1.5) [107]. Mutagenesis studies revealed two key amino acids within exon A that are required for this effect, Asp$^{578}$ and Lys$^{585}$, which are part of the Ca$^{2+}$-binding loop of CBD2 [108] (see Figure 3). It seems plausible that these two residues are required to maintain a conformation of CBD2 that is competent to bind Ca$^{2+}$. Whether the pH effect on Na$^{+}$-dependent inactivation occurs via CBD2, the XIP region, or elsewhere in the NCX1 molecule, remains unclear.

It would seem that regulation of NCX1 function by Na$^+$, Ca$^{2+}$ and PIP$_2$ as described above is in general poised to enhance activity of the exchanger in promoting Ca$^{2+}$ extrusion and reducing Ca$^{2+}$ entry modes of operation. Furthermore, these regulatory influences seem too slow to regulate the exchanger significantly during the time course of one heart beat, but are more likely to respond integratively to longer-term homeostatic influences [109]. Thus, for example, ischaemic conditions that result in elevated intracellular Na$^+$ and reduced ATP levels would be expected to shift NCX1 into the reverse mode of operation both thermodynamically and kinetically. These conditions, however, lead to regulatory inactivation of the exchanger, hence minimizing Ca$^{2+}$ entry. In contrast, raising cytosolic Ca$^{2+}$ will activate the forward operation of NCX1 thermodynamically, kinetically and via the dual regulatory influences of Ca$^{2+}$ activation and the relief of Na$^+$ inactivation. Thus a developing theme is the functional and mechanistic interconnectivity between the different regulators of NCX1 activity: Ca$^{2+}$, Na$^+$, PIP$_2$, and pH. The recent determination of the CBD structures [59,60] provides an opportunity to advance our understanding of the mechanism underlying these related modes of NCX1 regulation to the molecular level.

The interaction of NCX1 with partner proteins has emerged recently as another critical modulatory influence on function. That the exchanger might interact with other proteins was first suggested following studies that demonstrated a specific localization of NCX1 to T-tubule membranes in cardiomyocytes [110], to plasma membrane domains overlying SR elements in smooth muscle cells [111], and on the basolateral membrane of renal epithelial cells [112]. An association between NCX1 and the cytoskeletal protein ankyrin was subsequently observed in vitro [113]. More recently, analysis of cardiomyocytes from ankyrin B-knockout heterozygous mice revealed decreased stability, reduced expression and mislocalization of NCX1 [114,115]. Presumably this arises from disorganization of the cytoskeletal network owing to reduced levels of ankyrin expression. As ankyrin is capable of binding to multiple proteins and linking them to the cytoskeleton, ankyrin interactions may underlie the observations that NCX1 is part of a macromolecular complex [70,71]. Such a complex may help co-ordinate Ca$^{2+}$ signalling in cardiac myocytes and other cells. The site of ankyrin binding to NCX1 has not yet been identified, but one might anticipate that it would be found in the large central cytosolic loop of the protein. Importantly, an interaction within this region has the potential to have significant influences on NCX1 function and its regulation above and beyond localization.

An interaction of NCX1 with the other cytoskeletal proteins has also been investigated. In particular, there is evidence that an interaction with the filamentous actin network influences both cell-surface delivery of NCX1, as well as playing an important role in modulating regulation via the central cytoplasmic loop [116,117]. Whether these effects of actin involve a direct protein interaction, or a more indirect one via a larger macromolecular complex, remain unknown.

Caveolins are small lipophilic scaffolding proteins identified as forming a coat structure on the cytoplasmic side of plasma membrane invaginations known as caveolae [118,119]. A variety of Ca$^{2+}$-sensitive signalling molecules cluster together in caveolae, possibly leading to integrated actions involved in Ca$^{2+}$ homeostasis [120,121]. Caveolae are enriched in cholesterol and sphingolipids, and hence these structures, and the caveolin proteins associated with them, can also be isolated biochemically as ‘lipid rafts’. The caveolin-3 isoform is expressed in heart, preferentially present on the surface membrane of ventricular myocytes, where it appears to play a key role in the regulation of normal cardiac function [121,122]. Although NCX1 is mostly present in the T-tubule membrane, a significant fraction of these two molecules overlap [123], and caveolin-3 and NCX1 have been shown in some studies to co-associate [124,125]. Other studies, however, do not support this association [126]. The reason for these different results is unclear, but might reflect different conditions or animal models. In addition to the situation in heart, NCX1 in neuronal cells has been shown to associate, at least partially, with caveolin-1 and -2 [127].

The number of protein partners shown to interact with NCX1 has increased dramatically in the last few years. PLM (phospho-lumen) is one of the better characterized of these [128]. PLM is
a member of the so-called ‘FXYD’ family of transport regulators, best known as modulators of Na\(^+\), K\(^+\)-ATPase activity in various tissues [129]. When phosphorylated at Ser\(^{68}\) by PKA (protein kinase A), the C-terminal cytosolic tail of PLM binds to two regions of the NCX1 cytosolic loop, inhibiting NCX1 function [130–132]. This would be parsimonious with the overall influence of \(\beta\)-adrenergic agonists to promote Ca\(^{2+}\) entry, SR loading and the contractility of cardiomyocytes. Consistent with these findings, PLM-null mice exhibit enhanced NCX1 function [133]. PLM has also been demonstrated to have an activating influence on cardiac Na\(^+\),K\(^+\)-ATPase [134]. As NCX1 and the Na\(^+\).K\(^+\)-ATPase are thought to co-associate in a macromolecular complex within the T-tubule [70], co-ordinate regulation of these functionally linked transporters may well be of physiological importance.

Two more recently discovered NCX1-interacting partners, both identified using yeast two-hybrid screening strategies, were calcineurin [135] and the 14-3-3 proteins [136]. In both instances, the interactions were inhibitory. Binding of the Ca\(^{2+}\)-sensitive phosphatase, calcineurin, to the cytosolic loop of NCX1 was enhanced in phenylephrine-induced hypertrophy, and may be responsible, at least in part, for the observed decrease in NCX1 activity observed under these conditions [135]. The 14-3-3 proteins are a large family of proteins implicated in a variety of signalling cascades as a result of their property of promiscuous binding to phosphorylated serine and threonine residues in target proteins [137]. Several different 14-3-3 protein family members are able to bind to the cytosolic loops of NCX1, NCX2 and NCX3 in a phosphorylation-dependent manner, inducing inhibition of activity [136]. The physiological or pathophysiologcal consequences of 14-3-3 binding have yet to be defined.

Phosphorylation of NCX1 remains a topic of much controversy. Several studies have suggested that NCX1 function can be modulated by adrenergic stimulation, operating via PKA and/or PKC (protein kinase C), in both myocytes and recombinant systems [61,138–141]. Furthermore, there is evidence for direct phosphorylation of the NCX1 protein, when expressed heterologously [71,141]. However, further studies have revealed that, at least in certain cases, adrenergic activation of Na\(^+\)/Ca\(^{2+}\) exchange can be explained either by indirect effects on local substrate concentration or by effects on contaminating activities not related to the exchanger [142,143]. It remains possible that another contributing factor to this controversy is the different sensitivity of NCX1 in different animal species (particularly frogs) [144]. In addition, although activation of PKC has been shown in heterologous systems to result in both phosphorylation of NCX1 and stimulation of exchange activity [145], subsequent experiments demonstrated that the phosphorylation events were not related to changes in activity [146]. It remains possible, however, that NCX1 activity is regulated indirectly, via its participation in macromolecular complexes with other proteins, including PLM [70,71,128]. Extensive experiments in squid axon, where phosphorylation of a small regulatory protein stimulates exchanger activity, are supportive of such a model [86].

**NCX1 AND PHYSIOLOGY**

The role of the Na\(^{+}\)/Ca\(^{2+}\) exchanger in normal cellular physiology has in the past either been inferred from theoretical considerations and from examining its function in isolation under artificial conditions or has been determined by employing crude ion substitution experiments or by using inhibitors of doubtful selectivity (such as benzamil) [9]. Although these approaches have allowed the broadly based conclusion that a major role for the exchanger is in the removal of Ca\(^{2+}\) from cells, the nature of the invasive approaches has precluded detailed study, particularly under physiological conditions. More recently, the generation of more selective inhibitors and, most importantly, genetic approaches, has allowed new fascinating insights [6,147].

In the heart, contraction is initiated by a small influx of Ca\(^{2+}\) across the plasma membrane that then induces a larger release from the SR. The relative magnitude of these two components differs from species to species, ranging from less than 10 % influx in rodents to approx. 30 % influx in humans [68]. Relaxation requires Ca\(^{2+}\) removal and sequestration in proportion with prior influx and release, in order to maintain long-term cellular Ca\(^{2+}\) homoeostasis. Measured activity levels of Na\(^+\)/Ca\(^{2+}\) exchange compared with plasma membrane Ca\(^{2+}\)-ATPase clearly indicated that the exchanger was the predominant Ca\(^{2+}\) efflux pathway and therefore essential to cardiac Ca\(^{2+}\) homoeostasis and hence function [9]. Ca\(^{2+}\) extrusion via the exchanger also has the secondary consequence of generating a depolarizing current (carried by Na\(^+\) ions) which may contribute to the shape and length of the action potential. In addition, theoretical considerations suggest that at the peak of the action potential, thermodynamic conditions favour Ca\(^{2+}\) entry via NCX1. The extent to which this Ca\(^{2+}\) entry contributes to excitation–contraction coupling in myocytes has been controversial, since, among other things, the precise concentration of ions under the plasma membrane experienced by the exchanger is uncertain [68]. These uncertainties speak of the difficulties in distinguishing NCX1 function under physiological conditions.

The critical nature of NCX1 activity to cardiac function was originally supported by studies in mice where global knockout of the NCX1 gene had an embryonically lethal phenotype at approx. 8.5 days post-conception, which was attributed to a cardiac defect [148–151]. More recent studies, however, suggest that the cause of lethality in NCX1-knockout mice may be extra-cardiac in origin [152]. Still, loss of NCX1 function during development cannot be compensated for by other members of the NCX family or by other Ca\(^{2+}\) transport mechanisms. Surprisingly, cardiac-specific knockout of NCX1 was not lethal [153]. Only a modest deficit in cardiac function was present in young animals, although this did progress to the development of cardiac hypertrophy and ultimately heart failure as the animals aged.

Myocytes from these knockout animals display remarkably normal Ca\(^{2+}\) dynamics. More detailed examination revealed that the knockout myocytes have a dramatically reduced L-type Ca\(^{2+}\) channel current (although there was no loss of Ca. 1.2 protein), that could be normalized upon dialysis with the Ca\(^{2+}\)-chelator BAPTA [bis-(α-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid] [154]. This result suggests that reduced Ca\(^{2+}\) clearance from the diadic cleft in the absence of NCX1 leads to enhanced Ca\(^{2+}\)-dependent inactivation of the L-type Ca\(^{2+}\) channel in the knockout myocytes. Additionally, the myocytes had a dramatically shortened action potential due to up-regulation in the hyperpolarizing I\(_{Ks}\) (outward K\(^{+}\) current), and an increase in expression of K\(^{+}\) channel subunits K,4.2 and KChIP (K\(^{+}\) channel-interacting protein) [155]. The combined effect of a much shorter action potential and a rapidly inactivating Ca\(^{2+}\) current was that knockout myocytes had an 80 % reduction in the flux of Ca\(^{2+}\) across the sarcolemma. The fact that this dramatic reduction in trigger Ca\(^{2+}\) did not result in reduced SR Ca\(^{2+}\) release and reduced contractility was due to an increase in the gain of excitation–contraction coupling. The mechanism that underlies this change in gain is currently not known. Ultimately, however, operating at maximal gain prevents changes in Ca\(^{2+}\) homoeostasis needed to respond to increases in demand for cardiac output. Consequently, this deregulation of
integrated Ca\(^{2+}\) transport leads in the long term to hypertrophy and heart failure.

Interestingly, examination of transgenic mice that overexpress NCX1 demonstrate precisely the converse alterations in sarcolemmal properties. Namely, an increase in the L-type Ca\(^{2+}\) current and an elongation of the action potential plateau phase [156]. This fascinating and remarkable series of compensatory changes clearly illustrates the finely tuned and highly interdependent network of protein molecules responsible for overall Ca\(^{2+}\) homeostasis in heart cells. Presumably, changes in local [Ca\(^{2+}\)]\(_i\) itself, or possibly alterations in multimeric protein complexes associated with the NCX1 protein, underlie the adaptations to large shifts in NCX1 protein expression in these genetically altered myocytes.

The extent to which the exchanger can reverse during the action potential to deliver trigger Ca\(^{2+}\) for further SR release has been a subject of considerable controversy [68]. New data from cardiac-specific NCX1-knockout mice suggest that normally reverse exchange activity at the peak of the action potential helps to load the diadic cleft, and hence synchronize rapid SR release [157]. In the absence of NCX1, Ca\(^{2+}\) does not build up as quickly. Instead, the lack of NCX1-driven Ca\(^{2+}\) removal leads to a slower and larger build up of diadic Ca\(^{2+}\), and hence a slight delay in SR Ca\(^{2+}\) release.

The role of the exchanger in other tissues has not been examined in the same detail as it has in the heart [9]. In brain, crude ion substitution experiments as well as the high density of expression of the Na\(^+\)/Ca\(^{2+}\) exchanger localized to sites of high plasmalemmal Ca\(^{2+}\) flux, such as presynaptic terminals, have all led to the main conclusion that the role of the exchanger in neurons is to extrude Ca\(^{2+}\) following its entry, hence terminating signalling events [49,158]. The role of the exchanger in brain neurons is complicated further by the expression of at least two NCX subtypes here, NCX1 and NCX2, as well as lower and more selective expression of NCX3 [49,159]. The widespread high level of NCX2 expression implies a key role in neuronal Ca\(^{2+}\) homeostasis and function (see below). There is also evidence that NCX3 plays a selective and critical role in protecting cerebellar granule cells from ischaemic insults [56,160]. Further details regarding the localization of NCX1, NCX2 and NCX3 expression in different neurons, as well as the precise subcellular distribution of the three proteins, will provide insight into possible distinct physiological roles for each of these different gene products [161].

The Ca\(^{2+}\) extrusion mode of the Na\(^+\)/Ca\(^{2+}\) exchanger generates an inward, depolarizing, current as a consequence of the uneven charge translocation during turnover. When coupled to the extrusion of Ca\(^{2+}\) due to a signal initiated by entry across the plasma membrane, this depolarization can have a role in shaping an action potential, or can even induce a feedback effect on the entry pathway (reducing the driving force for Ca\(^{2+}\) entry). The inward Na\(^+\)/Ca\(^{2+}\) exchanger current due to efflux of Ca\(^{2+}\) released from intracellular stores is an important component of downstream signalling, by modulating other voltage-gated channels. For example, serotonin and orexin, acting via their receptors to release Ca\(^{2+}\) from intracellular stores, are thought to depolarize their target neurons, at least in part, via this mechanism [162–164].

As discussed above, relatively subtle alterations in either membrane potential or ion concentration close to the membrane can induce the Na\(^+\)/Ca\(^{2+}\) exchanger to reverse operation and catalyse the influx of Ca\(^{2+}\) instead of its extrusion. This mode of operation is thought to contribute significantly to Ca\(^{2+}\) influx induced by, among other signals, the neurotransmitter glutamate, presumably as a consequence of Na\(^+\) entry through ionotropic glutamate receptors [165,166]. These actions of the Na\(^+\)/Ca\(^{2+}\) exchanger are unlikely to play a major role during the course of a single action potential, but are more likely to be important in slower processes or longer-term cumulative effects. Repetitive rapid action potential trains, as found in some neurons, could lead to persistent elevation in Na\(^+\), which via its influence on Na\(^+\)/Ca\(^{2+}\) exchanger function, would induce a longer-term modulation of [Ca\(^{2+}\)]. This complexity of roles for a Na\(^+\)/Ca\(^{2+}\) exchanger in brain neurons presumably reflects the molecular diversity of structures encoding Na\(^+\)/Ca\(^{2+}\) exchanger molecules, as well as the formation of protein complexes and functional coupling mediated by local ion gradients in restricted diffusional spaces.

The NCX1 gene product is relatively abundant in a variety of smooth muscles [167]. Indeed, altering Na\(^+\)/Ca\(^{2+}\) exchanger activity can have a large effect on subsequent agonist-induced contraction [168–170]. However, few studies have been able to demonstrate a reliable correlation between inhibition of Na\(^+\)/Ca\(^{2+}\) exchanger function and changes in cytosolic Ca\(^{2+}\). Subcellular localization of NCX1 in arterial smooth muscle cells revealed that the protein was present in domains of the plasma membrane associated with underlying SR elements [111]. The Na\(^+\)/Ca\(^{2+}\) exchanger therefore has a privileged indirect communication with the SR stores, operating through a highly restricted diffusional space between the plasma membrane and junctional SR elements. Thus myogenic tone and subsequent agonist-induced contraction can be enhanced by treatment with relatively low doses of ouabain, acting to inhibit its target, the Na\(^+\)-K\(^+\)-ATPase [9]. This inhibition raises sub-sarclemmal [Na\(^+\)], which in turn inhibits extrusion of Ca\(^{2+}\) through the Na\(^+\)/Ca\(^{2+}\) exchanger. Under these conditions of reduced plasma membrane efflux, entering Ca\(^{2+}\) is sequestered into the SR stores, leading to an enhanced contractile response.

Many agonists stimulate sustained contraction through the activation of receptor- and store-operated channels that allow both Ca\(^{2+}\) and Na\(^+\) entry. Inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger under these conditions dramatically reduces agonist responsiveness, suggesting that reverse-mode operation of the exchanger stimulated by elevated Na\(^+\) entry through the store-operated channels, is critical for Ca\(^{2+}\) entry, store loading and contraction [168–170]. Despite the fascinating localization of NCX1 demonstrated in smooth muscle cells, and its importance to the physiological role of the exchanger, little specific information has emerged regarding the molecular mechanism for restricted localization, nor details of the macromolecular complex which presumably exists in smooth muscle cells, as well as in other tissues.

The Na\(^+\)/Ca\(^{2+}\) exchanger plays a special role in Ca\(^{2+}\) homoeostasis in the kidney. The majority of Ca\(^{2+}\) filtered at the glomerulus is reabsorbed passively along the proximal nephron and in the loop of Henle. However, control of the final few percent of Ca\(^{2+}\) reabsorption within the distal nephron is a key point of regulation. It is at this site that hormones such as vitamin D, and parathyroid hormone have their action, co-ordinately regulating the apical entry, cytosolic binding and basolateral efflux components of the transepithelial Ca\(^{2+}\) transport machinery [171]. Distal nephron segments, particularly the connecting segment, express a very high level of NCX1 on their basolateral membranes [112], which helps to drive transepithelial active reabsorption of Ca\(^{2+}\). Thus, in the kidney, NCX1 is involved in the regulation of systemic Ca\(^{2+}\) levels, as opposed to regulating the intracellular level of [Ca\(^{2+}\)], as is the case in all other tissues in which it is expressed.

Gene-knockout technology has also been applied to the other NCX family members. NCX2-knockout mice display enhanced synaptic plasticity and a shift from long-term depression toward long-term potentiation at hippocampal synapses and enhanced performance in several hippocampal-dependent learning and memory tasks [7]. These results are consistent with a predominant role for NCX2 in both pre- and post-synaptic Ca\(^{2+}\) clearance. Larger presynaptic [Ca\(^{2+}\)] transients due to
the loss of NCX2 function will induce increased transmitter release, whereas similarly increased post-synaptic transients favour the Ca\(^{2+}\)-dependent phosphorylation events underlying LTP (long-term potentiation) [172,173]. Presumably other transport pathways account for longer-term Ca\(^{2+}\) homoestasis, which appears normal in these mice.

NCX3-knockout mice have a distinct pathology restricted to deficits in neuromuscular transmission as well as to increased necrosis of muscle tissue [174]. This finding is consistent with the known expression of NCX3 in skeletal muscle. It is not clear whether, in addition to the observed skeletal muscle defects, NCX3-knockout mice also display altered neuromuscular control as a result of loss of NCX3 expression in the cerebellum.

**NCX1 AND PATHOLOGY**

As a consequence of its central role in helping to orchestrate normal cellular Ca\(^{2+}\) homoestasis in a variety of tissues, the Na\(^+/\)Ca\(^{2+}\) exchanger is also a key player in pathological situations that involve dysregulation of Ca\(^{2+}\) balance. Cardiac arrhythmias are a leading cause of death in cardiovascular disease. NCX1 contributes to cardiac arrhythmias in two ways [175,176]. First, reduced forward-mode Ca\(^{2+}\) efflux, increased reverse-mode Ca\(^{2+}\) influx, or both during systole, mediated by events such as elevated intracellular Na\(^{+}\) or other pathogenic mechanisms, can shift the Ca\(^{2+}\) extrusion/sequestration balance, leading to SR Ca\(^{2+}\) overload. This causes an increase in both the sensitivity and the extent of SR Ca\(^{2+}\) release, generating so-called spontaneous release and extrasystolic events [177]. Secondly, the extra Ca\(^{2+}\) release increases subsequent NCX1 activity during diastole which will generate an inward current. This depolarizing current can contribute to an elongation of the action potential, or can be of sufficient magnitude to generate extra action potential events.

Heart damage or chronic hypertension can lead to cardiac hypertrophy which eventually results in heart failure. The genetic pathways that are initially activated under these pathological conditions result in an up-regulation of NCX1 [80]. There has been much discussion as to whether these compensatory changes are adaptive or maladaptive to heart function in the long run [178]. It is probable that the initial reprogramming is a response to increased contractile demand that leads to enhanced Ca\(^{2+}\) entry and consequent overloading of the SR. An increase in Na\(^+/\)Ca\(^{2+}\) exchanger extrusion activity will offset the increased Ca\(^{2+}\) entry to maintain Ca\(^{2+}\) homoestasis. Eventually, the associated depolarizing current may become arrhythmogenic [179]. Moreover, sustained hypercontractility may cause readjustment of SR Ca\(^{2+}\) transport to help reset content. At that point, enhanced Na\(^+/\)Ca\(^{2+}\) exchanger activity becomes maladaptive, unloading the SR stores which leads to contractile insufficiency and heart failure [180].

The Na\(^+/\)Ca\(^{2+}\) exchanger is also thought to play a key role in ischaemia/reperfusion injury. Although mechanistically complex with a variety of factors that are likely to contribute to the pathology, it seems likely that H\(^{+}\) ions that accumulate during the ischaemic period are removed during reperfusion, largely through the action of the Na\(^+\)/H\(^+\) exchanger. This transport step leads to a build up of intracellular Na\(^{+}\), which subsequently either reduces NCX1-mediated Ca\(^{2+}\) influx, or increases Ca\(^{2+}\) entry [9,181]. Either way, the consequence is SR Ca\(^{2+}\) overloading, and consequent spontaneous release and extrasystolic arrhythmias, as described above.

Action of the Na\(^+/\)Ca\(^{2+}\) exchanger has also been linked to pathology outside the heart. There is a lot of evidence suggesting that, during excitotoxic stimulation in brain, particularly during ischaemia, large changes in both membrane potential and ion gradients lead to unregulated Ca\(^{2+}\) entry and activation of a number of Ca\(^{2+}\)-sensitive degradative enzymes, such as the protease calpain, subsequently leading to neuronal degeneration [182,183]. The role of the exchanger in this process has been controversial. On theoretical grounds, one might anticipate that the collapse of the membrane potential and ion gradients would lead to reverse-mode Na\(^+/\)Ca\(^{2+}\) exchanger-mediated Ca\(^{2+}\) entry [184–186]. Under these conditions, inhibition of the exchanger would be expected to protect against ischaemic damage. Indeed, inhibition studies in white matter, and in neurons in vitro support such a role. On the other hand, at a later time, during potential recovery from ischaemia particularly in areas penumbral to the major injury, the Na\(^+/\)Ca\(^{2+}\) exchanger would be anticipated to be a major pathway for clearance of Ca\(^{2+}\) that had built up previously. Several animal studies support this neuroprotective role for the exchanger [187–189].

Evidence has been presented that the Na\(^+/\)Ca\(^{2+}\) exchanger in vascular smooth muscle cells plays an important contributory role to the increase in myotonic tone that underlies salt-sensitive hypertension [190]. Both pharmacological and genetic inhibition of NCX1 were protective against, while transgenic overexpression of NCX1 potentiated, the effect of high salt on hypertension. These data suggest that reverse-mode NCX1 Ca\(^{2+}\) entry, possibly in response to Na\(^{+}\) loading of vascular smooth muscle cells, plays a critical role in the development of salt-sensitive hypertension.

One of the common threads that emerges from examining the physiological and pathophysiological roles for the Na\(^+/\)Ca\(^{2+}\) exchanger is that altered membrane potential and/or changed ion gradients can lead to unregulated reverse-mode operation of the exchanger, Ca\(^{2+}\) overload and deleterious consequences. Forward-mode operation of the exchanger under these conditions is actually protective. Thus consideration of the Na\(^+/\)Ca\(^{2+}\) exchanger as a putative therapeutic target would require selective inhibition of reverse-mode, as opposed to forward-mode, operation. Coincidentally, over the course of the last 10 years, an expanding family of inhibitors with just these properties have emerged: KB-R7943, SEA0400 and SN-6 [147,191]. Directional inhibition of the exchanger under near equilibrium conditions is, of course, impossible as it violates thermodynamic principles of microreversibility and energy transfer. Apparent directional inhibition can occur far from equilibrium, where one set of conditions (for example those driving reverse-mode operation) favour binding of the inhibitor, while other conditions driving the opposite direction of transport disfavour inhibitor binding [192]. All three inhibitors noted above interact to promote the Na\(^{+}\)-dependent inactivation step of the NCX1 reaction cycle. The inactivated state can only be appreciably populated under conditions favouring reverse-mode operation (high cytosolic Na\(^{+}\); relatively low cytosolic Ca\(^{2+}\)), which thus provides a potential explanation for the apparent mode-selective directional inhibition by these compounds. Several previous studies have employed the reverse-mode inhibitors, and demonstrated their effectiveness in providing partial protection against ischaemic damage and hypertension [147,190,191]. These new findings have reinvigorated exploration of the NCX gene family members as targets for therapeutic intervention, and so new more effective and selective classes of inhibitors may be developed in the near future.

**THE SLC24 K\(^{+}\)-DEPENDENT Na\(^+/\)Ca\(^{2+}\) EXCHANGE FAMILY**

The protein product in the SLC24, or Na\(^+/\)Ca\(^{2+}\)-K\(^{+}\) exchanger, gene family to be identified was NCKX1 in the outer segments of rod photoreceptors [15]. Indeed, NCKX1 remains the only member of the family to have been extensively studied in its
endogenous setting [193]. Following the isolation and cloning of NCKX1 from bovine retina, the other family members, NCKX2–NCKX5, were identified by homology cloning or database mining [22–25]. Sequence comparison among the NCKX family gene products reveals that NCKX1/NCKX2 and NCKX3/NCKX4 pairs each share approx. 60% amino acid identity, whereas there is only approx. 40% identity between pairs. NCKX5 is slightly closer in sequence to the NCKX3/NCKX4 pair. In all cases the highest similarity is restricted to the hydrophobic regions of the proteins. A striking difference in length exists between NCKX1 (approx. 1200 amino acids) and the other family members (ranging in length from 500 to 670 amino acids), the extra sequence corresponding to regions of the two large hydrophilic loops in the protein (see below). The apparent size of the NCKX proteins observed on gels also varies with their size, glycosylation state and alternative splicing pattern: ~220 kDa for NCKX1, ~75 kDa for NCKX2, ~70 kDa for NCKX3, ~60 kDa for NCKX4 and ~50 kDa for NCKX5 [14,22–24] (X.-F. Li, F. Visser, G. Yang and J. Lytton, unpublished work). NCKX1, NCKX2 and NCKX4 all have a short segment of their cytosolic loop that is subject to alternative splicing. The role of this splicing event remains unknown, however.

The expression of NCKX1 is restricted almost exclusively to the eye, although its presence in cells of haemopoietic origin has also been reported [194–196]. NCKX2 expression is abundant in neurons throughout the brain, with particularly high levels found in deeper cortical layers, hippocampal pyramidal cells, specific thalamic nuclei, the pontine nucleus and the molecular layer and the deep nuclei of the cerebellum [22,195]. NCKX2 is also expressed in retinal ganglion cells and in cone photoreceptors [197,198]. NCKX3 and NCKX4 were also highly expressed in brain, with distinct patterns compared with NCKX2, particularly in cortex and thalamus [23,24,195]. These two genes were also more broadly expressed in tissues outside the brain. NCKX5 was most abundant in the eye and skin, but also expressed at a significant level in brain and thymus, and at much lower levels in other tissues [25]. In contrast with the studies on NCKX1, there are no reports which study the promoter regions of the NCKX gene family, or regulation of expression at the transcriptional level. Molecular cloning studies and database analysis have revealed some heterogeneity at the 5′-end of NCKX2 transcripts, suggesting multiple possible 5′-untranslated region exons, similar to those found in the NCX1 gene [22,199]. The significance of such alternative first exons in the NCKX2 gene remains unknown.

STRUCTURE OF NCKX PROTEINS

The overall topology of the NCKX proteins is identical between family members, and reminiscent of the NCX proteins (see Figure 5). An initial hydrophobic region is thought to be cleaved off as a signal peptide [200], followed by a relatively long extracellular domain containing at least one glycosylation site, a cluster of five proposed hydrophobic transmembrane spans, a larger cytosolic loop and finally a second cluster of hydrophobic transmembrane spans. Experimental evidence places the C-terminus of NCKX2, and by comparison all other NCKX family proteins, outside the cell [38,201]. This is in contrast with the available data for NCX1 which places the C-terminus inside the cell. Thus the C-terminal hydrophobic region may have a different orientation in the NCKX family compared with the NCX family. It is tempting to speculate that this might be related to their different ionic specificities (see below).

The two defining α-repeat regions in NCKX2 have been determined to have opposite orientations with respect to the membrane, much like in NCX1 [37]. There is, however, no evidence in NCKX2 for the re-entrant loop structures observed in NCX1. Proximity analysis using cross-linking experiments provided evidence that the two α-repeat regions are probably close together in the membrane. Just as with NCX1, however, the data were hard to reconcile with a purely helical model [39]. Functional analysis of the α-repeat regions by mutagenesis revealed that many amino acids have an impact on function and on the interaction with the transported ions [32]. Most of these appeared to influence Ca2+ and K+ binding in parallel, suggesting a general influence on the transport mechanism [202]. Several residues stood out from this analysis (see Figure 5). There were two key conserved acidic amino acids, glutamic acid in the first α-repeat and aspartic acid in the second, that were absolutely required for transport function. Further analysis has revealed an aspartic acid in the second α-repeat of NCKX family members that when altered to the corresponding asparagine residue found in the NCX family, rendered the NCKX family K+-independent [203]. Presumably this residue is critical in defining the K+-binding site.

NCKX TRANSPORT FUNCTION

Recent analysis comparing ion binding with the external facing transport sites between different NCKX family members has demonstrated a similar apparent affinity of approx. 1 μM for Ca2+ and approx. 30 mM for Na+ [204]. Na+ activation of transport displayed a co-operative interaction, consistent with three or more ions binding, whereas both Ca2+ and K+ displayed activation consistent with a single ion binding. In the case of K+, when analysed in the presence of Li+ as the counterion, NCKX2 displayed a dramatically lower apparent affinity of approx. 40 mM compared with approx. 1 mM for NCKX3 or NCKX4. Selectivity for other ions at the K+ site of NCKX2 was also significantly different, with both Cs+ and NH4+ stimulating transport to a greater extent than in NCKX3 or NCKX4 [205]. Mutation of the α-repeat residues that differ between NCKX2 and NCKX4 revealed that a threonine residue in the second α-repeat of NCKX2, lying just cytoplasmic to the key conserved aspartic acid, was responsible for the low apparent K+ affinity of NCKX2 [204]. The mutation of this single residue to the corresponding alanine residue in NCKX4 converted NCKX2 from low into high apparent K+ affinity. Less is known regarding ion binding from the cytoplasmic side of the membrane, although some studies in rod outer segments suggest that both Ca2+ and K+ interact with similar apparent affinity to their binding sites from both sides of the membrane [206]. The physiological significance of the difference in apparent K+ affinity between isoforms is not obvious, but may relate to differential localization of the isoforms at sites of [K+] fluctuation.

REGULATION OF NCKX FUNCTION

Information on regulation of NCKX family members is relatively under-developed compared with what is known for NCX1. Activity of the latter is regulated via the central cytosolic domain, and so it seems reasonable to begin exploring NCKX regulation by examining the corresponding region of those proteins. Unlike the NCX family, there is little sequence similarity in the central cytosolic loop of NCKX family members. Both NCKX1 and NCKX2 have a short amphipathic region at the N-terminal end of the central cytosolic loop, but it does not resemble the XIP region of NCX1 significantly. In addition, just downstream of this region, NCKX1 and NCKX2 share a short stretch of amino acid similarity surrounding a possible CaMKII (Ca2+-/calmodulin-dependent protein kinase) phosphorylation consensus motif [22].
Neither of these regions has been examined experimentally to see whether it serves a regulatory role.

The NCKX1 cytosolic loop contains an unusual repeated acidic amino acid motif. Although this general feature is found in common between NCKX1 gene products from different species, the length of the motif, its sequence and the number of times it is repeated are not conserved among rats, cows or humans [196]. A similar acidic repeat motif (although not of similar sequence) is present in a subunit of the cGMP-gated channel [207], which is found to associate with NCKX1 in a molecular complex in rod photoreceptors [208]. The role of the acidic motifs in complex formation has not been tested. The NCKX1/cGMP-gated channel complex may be important for the co-ordinated regulation of activity (Thr166, Thr476 and Ser504) and a cysteine residue (Cys395) thought to be important for redox regulation of NCKX2.

Measurements of NCKX2 function in heterologous systems typically involve loading cells with Na\(^+\) to various extents and then following Ca\(^{2+}\) entry with a fluorescent dye trapped in the cytoplasm. When a gramicidin-clamp was used to control the [Na\(^+\)] at defined levels, a time-dependent inactivation process was revealed that required high levels of intracellular [Na\(^+\)], apparently via a mechanism involving binding to the transport sites [211]. This process is reminiscent of the Na\(^+\)-dependent inactivation that has been well described for NCX1 (see above).

Regulation of NCKX function by phosphorylation via PKC has been demonstrated recently for NCKX2, but was not observed in either NCKX3 or NCKX4 [212]. The neurotransmitter glutamate acting through synaptic metabotropic receptors would be expected to induce a similar PKC-mediated activation [213,214]. Indeed, activation of NCKX transport by phorbol ester could be demonstrated at the large presynaptic terminal, the calyx of Held. Treatment of NCKX2-transfected HEK-293 cells with phorbol ester resulted in significant stimulation of exchange activity, which could be blocked by overexpression of dominant-negative PKCs. Stimulation of activity correlated with an increase in the level of incorporation of \(^{32}\)Pphosphate into immunoprecipitated NCKX2, and could be blocked by mutation of multiple potential PKC phosphorylation sites on NCKX2, but not by any one single mutation (see Figure 5). These data suggest a complex model of regulation involving direct phosphorylation of NCKX2. These new modes of regulation of NCKX2 may play an important role in mediating complex integration of Ca\(^{2+}\) at synaptic sites, where rapid changes in Ca\(^{2+}\) flux play important roles in the control of neurotransmitter release and in the development of synaptic plasticity.

**NCKX AND PHYSIOLOGY**

NCKX transport function has been best described in the rod photoreceptor outer segments, where NCKX1 is the principal...
means for Ca\textsuperscript{2+} extrusion [193]. Here, in the dark, cGMP-gated channels are largely open, which results in a depolarizing Na\textsuperscript{+} entry as well as Ca\textsuperscript{2+} influx that is matched by NCKX1-mediated eflux. Under these conditions, the thermodynamic and kinetic properties of an NCKX-type transporter are critical for the maintenance of Ca\textsuperscript{2+} homeostasis. Upon stimulation with light, cGMP is hydrolysed, the cGMP-gated channels close, the resulting hyperpolarization triggering a change in signalling at the photoreceptor synapse, and NCKX1 activity drives Ca\textsuperscript{2+} down to a very low level. This change in [Ca\textsuperscript{2+}] is a key mediator of visual adaptation, resulting in an increase in cGMP synthesis and re-opening of the cGMP-gated channels under continual illumination [207,215]. NCKX2 likely plays a corresponding role in cone photoreceptors [198].

Discovery of the other NCKX family members originated in molecular cloning work, and so functional knowledge has lagged behind structural information. Recent findings, in axonal termini from the posterior pituitary, as well as at the giant synapse of the calyx of Held, have demonstrated a prominent role for K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in Ca\textsuperscript{2+} extrusion, particularly when intracellular [Ca\textsuperscript{2+}] is high [3,4,216]. In addition, studies on hippocampal neurons demonstrate a large component of NCKX-mediated Ca\textsuperscript{2+} flux [217]. On the basis of similar considerations of thermodynamics, kinetcs and the parallel to the rod photoreceptors, it seems likely that NCKX expression at these sites allows the maintenance of Ca\textsuperscript{2+} extrusion and homeostasis in neuronal environments which experience large and frequent ionic fluctuations across the plasma membrane. None of these studies, however, could distinguish which of the possible NCKX isoforms present (NCKX2, NCKX3 or NCKX4) was responsible for the activities measured.

The NCKX5 gene was recently found to have an unexpected role in the development of pigmentation in skin and retinal epithelia [25]. Analysis of the ‘golden’ phenotype in zebrafish identified a mutation in the NCKX5 gene resulting in impaired skin pigmentation. This defect could be partially rescued by reintroduction of wild-type zebrafish or human NCKX5 genes. Furthermore, analysis of the SNP (single nucleotide polymorphism) databases revealed a striking association of a single coding region polymorphism in NCKX5, changing a highly conserved amino acid to a threonine residue, with humans of Northern European origin, in comparison with those of African or Asian origin. Analysis of the NCKX5 protein in melanoma cells suggests that it associates with mature melanosomes [25,218]. This would be a unique cellular localization for NCKX5, as other members of the NCKX family are found at the plasma membrane. It has been hypothesized that, in the melanosome, NCKX5 may be coupled to the proton gradient generated by the V-ATPase (vacuolar-type ATPase) via a Na\textsuperscript{+}/H\textsuperscript{+} exchanger and thus drive Ca\textsuperscript{2+} uptake [25]. However, the role for the NCKX5 protein or for Ca\textsuperscript{2+} transport in melanosome biogenesis and maturation is not known. Neither is there any information concerning the impact on function of the polymorphism associated with European populations. Furthermore, significant levels of NCKX5 mRNA have been found in brain and thymus, although neither localization nor protein expression has been investigated in those tissues [25]. NCKX5 function is thus a fertile and exciting new area of investigation.

Although the physiological role for NCKX1 in rod and NCKX2 in cone photoreceptors, and the probable role for NCKX5 in melanocytes has become clear over the last few years, specific and distinct roles for NCKX2, NCKX3 and NCKX4 are only beginning to be unravelled. These three gene products are widely expressed in brain neurons, along with exchangers of the NCX family, NCX1, NCX2 and NCX3. Distinct patterns of neuronal expression have been described for all of these gene products, although no clear physiological insight has yet emerged from those studies [195]. In addition to brain, functional evidence is beginning to emerge for an important role of NCKX-type exchangers in other cells and tissues, such as olfactory epithelium [219], vestibular sensory cells [220], smooth muscle cells [168] and spermatozoa [221].

More recently, the NCKX2 gene has been knocked out in mice with interesting consequences [8]. These animals display a clear deficit in neuronal Ca\textsuperscript{2+} transport and no overt compensatory increases in other known Ca\textsuperscript{2+} extrusion or sequestration pathways. However, there is a dramatic loss of LTP and a gain in long-term depression at hippocampal Shaffer collateral/C1 synapses. This result is anticipated for the loss of a postsynaptic extrusion mechanism, leading to a sustained Ca\textsuperscript{2+} elevation that shifts the balance from kinase to phosphatase activation [172,173]. The phenotype of the NCKX2-knockout mice is also in marked contrast with that of the NCX2-knockout mice [7]. The differences may in part be due to pre- compared with postsynaptic expression, but may also reflect kinetic differences in operation, such that NCX2 knockout predominantly affects the magnitude of the initial Ca\textsuperscript{2+} transient, whereas NCKX2 knockout predominantly influences the sustained Ca\textsuperscript{2+} response. Such a dramatic change in function in cells that express more than one Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger gene product clearly demonstrates the non-redundant and distinct actions of the different transporters. Although the effect of NCKX2 knockout on synaptic plasticity in the hippocampus was dramatic, the influence of these cellular changes on behaviour was relatively subtle. The NCKX2-knockout mice displayed no overt behavioural abnormalities, including classical tests of motor skill and memory. There was, however, a clear deficit in performance on specific tests of motor learning and memory that depended upon the benefit of prior experience. It will be exciting to continue investigating the mechanisms that underlie these distinct functional deficits in NCKX2-knockout mice and to anticipate specific changes resulting from knockout of the other NCKX gene family members.

**NCKX AND PATHOPHYSIOLOGY**

To date, no genetic disease has been clearly linked by either inheritance or association with any Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger gene in either the NCX or NCKX family. There is a significant ongoing effort to identify genetic causes of retinal disease, including screening for possible mutations in NCX1 and NCKX2 [199]. Several polymorphisms were identified in this study, some of which were considered to be pathogenic, especially in NCKX1. However, no definitive linkages could be confirmed.

The question of whether NCKX isoforms are neuroprotective in the face of ischaemic and/or excitotoxic insults has recently been a focus of several studies. Following a short period of ischaemia, ion gradients and membrane potential collapses precipitously, pH drops and uncontrolled glutamate release activates further cation fluxes [222]. These are all changes which, in principle, might be sufficient to reverse the direction of even NCKX family members. Studies using in vitro cultures exposed to altered ionic conditions, while inhibiting various Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activities supports a contributing role for both NCX and NCKX gene products to excitotoxic Ca\textsuperscript{2+} accumulation and neuronal damage [186,223]. On the other hand, animal models examining changing levels of expression and application of siRNA (small interfering RNA) to knockdown expression of selective NCX and NCKX members supports a protective role for NCKX2 in the development of ischaemic injury [224]. Clearly, further careful studies will be required to resolve these issues, and to define a time course for the relative action of different Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers in the
development of ischaemic damage. This is an important area of study that may provide new targets for therapeutic intervention in excitotoxic disease states.

THE CCX EXCHANGER FAMILY

The CCX branch of the CaCA exchanger superfamily contains one mammalian member, so called NCKX6, or NCLX [26,27]. This most recently cloned member of the family, which is widely expressed at moderate levels, was initially mis-classified as part of the NCXK branch. However, recognition that its sequence was significantly more divergent than other NCXK family members led to a more detailed phylogenetic study and reclassification [17]. Membership in a distinct clade of the CaCA superfamily tree implies that NCKX6/NCLX probably has distinct endogenous substrate specificity. Indeed, Palty et al. [27] suggested that Li⁺ can substitute for Na⁺ in this exchanger, to mediate K⁺-independent Li⁺/Ca²⁺ exchange. It seems unlikely, however, that Li⁺ is the endogenous substrate for this transporter. Intriguingly, full-length NCKX6/NCLX is retained in an endoplasmic reticulum-like compartment when expressed in HEK-293 cells [26]. If this is its endogenous site of expression (as is the case for NCKX5), NCKX6/NCLX may play a role in loading a novel Ca²⁺ store. An intriguing finding of studies on recombinant NCKX6/NCLX was that partial-length truncated proteins, containing only a single copy of the α-repeat, appeared to be functional [26,27]. This phenomenon of active partial length exchangers has also been reported for NCX1 [225,226]. Dimerization of these proteins clearly warrants further examination. (partially) active transporter. The new class of CCX transporters truncated half NCKX6/NCLX molecule, combines to form a superfamily [40]. In the latter model, either the truncated half in a single polypeptide of the CaCA superfamily have opposite orientations with respect to the membrane [228], it is unclear how a homodimer of a half protein would be functional. Possible explanations might involve an unidentified endogenous partner, and/or the concept of dual-topology precursors to the CaCA superfamily [40]. In the latter model, either the truncated half NCKX6/NCLX molecules are competent to incorporate into the membrane in two orientations and then associate as dimers, or a second unidentified partner, in the opposite orientation to the truncated half NCKX6/NCLX molecule, combines to form a (partially) active transporter. The new class of CCX transporters clearly warrants further examination.

CONCLUDING REMARKS

Since the initial molecular cloning of NCX1 and NCKX1 in the early 1990s, there has been a large expansion of structural information regarding Na⁺/Ca²⁺ exchangers, resulting in the identification of the CaCA superfamily, and a realization that Na⁺/Ca²⁺ exchanger function is encoded by a complex array of different gene products. The NCX1 protein of cardiac myocytes is clearly the best studied of these, yet even this protein yields new surprises every year. Similarly, members of the NCKX family continue to surprise with their much broader and varied roles than originally appreciated. The recently published proceedings from the Fifth International Conference on Sodium–Calcium Exchange are an excellent source of current information [229]. Full characterization of the complex functional and regulatory properties of all the different proteins, and a clearer understanding of the physiological role(s) for each, is a work in progress. These studies are anticipated to reveal intriguing new distinct roles for different Na⁺/Ca²⁺ exchanges in the control of Ca²⁺ transport and homeostasis in a variety of cellular environments.

I thank Dr Frank Visser (University of Calgary) for helpful comments and assistance with some of the Figures, as well as Mark Hilge (Radboud University of Nijmegen, Nijmegen, The Netherlands) for supplying molecular co-ordinates and assistance with the preparation of Figure 3. Work in my laboratory has been supported by the Alberta Heritage Foundation for Medical Research and the Canadian Institutes of Health Research.

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Received 10 May 2007/6 June 2007; accepted 7 June 2007
Published on the Internet 29 August 2007, doi:10.1042/BJ20070619

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